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Isolation and Molecular Typing of *Naegleria fowleri* from the Brain of a Cow That Died of Primary Amebic Meningoencephalitis

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***Naegleria fowleri* causes an acute and rapidly fatal central nervous system infection called primary amebic meningoencephalitis (PAM) in healthy children and young adults. We describe here the identification of *N. fowleri* isolated from the brain of one of several cows that died of PAM based on sequencing of the internal transcribed spacers, including the 5.8S rRNA genes.**

Naegleria fowleri, an ameboflagellate, has been known to cause an acute, fulminant, and rapidly fatal central nervous system infection called primary amebic meningoencephalitis (PAM) in previously healthy children and young adults with a recent history of exposure to warm fresh water (5). Although a large number of animals besides humans also bathe, swim, and frolic in thermally polluted waters, only a single case of PAM in a South American tapir has been described (4). Recently, Daft et al. (1) reported PAM in Holstein cattle based on immunofluorescent identification of *N. fowleri* in brain sections, supporting the fact that PAM is not restricted to humans. In this report, we describe the isolation into culture of an ameba from the brain of one of the infected cows and identify the ameba as *N. fowleri* based on morphology and sequencing of the internal transcribed spacers (ITS), including the 5.8S rRNA genes.

Specimens from six Holstein heifers with clinical signs of anorexia, facial paralysis, circling, ataxia, and convulsions were received by the California Animal Health and Food Safety Department in the summer of 1999 (1). These animals belonged to a cattle ranch in San Bernardino, CA. Brain tissue exhibited lesions in ventral meninges, cerebellum, and olfactory lobes. *N. fowleri* trophozoites were demonstrated in the lesions by immunofluorescence (7). No gross lesions were seen in other organs (1).

An agar plate inoculated with fresh brain pieces from a cow that died of meningoencephalitis was incubated at 42°C. Since the plate was contaminated with fungi, the amebae were migrated successively on several other agar plates by cutting out a small piece of agar containing a few amebae in order to get rid of the fungal contaminants. Amebae free of fungal contamination were grown in 10 ml of modified Nelson's medium containing 10% fetal bovine serum, 100 μg/ml gentamicin, and 100 μg/ml imipenem (Merck & Co., Inc., West Point, PA) and incubated at 37°C (6). After about 2 h, when the amebae were found attached to the surface of the flask, the medium in the flask was gently swirled and removed and fresh medium as above was introduced. After two such manipulations, the cultures were allowed to incubate for 2 days, and about 1 ml (after vigorously shaking the flask) was removed and transferred to fresh medium containing the fetal bovine serum but without antibiotics, as before. Aliquots were also inoculated into brain heart infusion, sheep blood agar plates.

DNA was isolated from pelleted trophozoites of the strain isolated from cow brain using the UNSET method (3). The ITS1, 5.8S, and ITS2 rRNA genes were PCR amplified, and the PCR product was sequenced (both strands) without cloning using the ITS forward primer and the ITS reverse primer, corresponding to the 3′ end of the small subunit rRNA and the 5′ of the large subunit rRNA, respectively (2). Sequencing was performed using a Beckman CEQ2000 sequencer using the CEQ Dye Terminator cycle sequencing kit (Beckman Coulter Inc., Fullerton, CA). The sequence was compared to those of the different *N. fowleri* types that are published.

Amebae grew in the axenic medium and produced, occasionally, flagellates with two flagella exhibiting gyrating movement. The axenically growing amebae from the brain were designated as CDC: V 444 and examined with a microscope equipped with differential interference contrast optics. Sterility tests indicated that the culture had become axenic, as no viable bacteria were recovered.

Amebae exhibited eruptive movement by producing hemispherical bulges of hyaline cytoplasm, a feature characteristic of the genus *Naegleria*. The amebae moved in a sinuous fashion and possessed a single nucleus with a centrally placed large nucleolus. The posterior ends of the amebae, especially those growing on agar plates, possessed a uroid, often with trailing filaments. The trophozoite measured from 8 to 22 μm, with a mean of 15 μm, based on the measurements of at least 20 trophozoites. The amebae encysted readily on 2- to 3-day-old agar plates; the cysts measured 7 μm to 14 μm, with a mean of 10 μm, based on the measurements of 10 cysts. They were smooth walled with one to three pores and flush with the cyst wall, and they possessed a single nucleus with a large nucleolus. Amebae that were washed with ameba saline and the pellets that were transferred to flasks containing ~10 ml distilled water and incubated at 37°C produced flagellates. Flagellates

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were visualized within 10 min, and by about 1 h more than 75% of the amebae had transformed into flagellates, each with two flagella. The flagellates moved either jerkily or in a circular or spiral motion. They measured from 6 to 14 μm, with a mean of 11 μm, based on the measurements of six flagellates. These morphological features clearly indicate that the amebae belonged to the genus *Naegleria*. Further, based on its reactivity with the monoclonal antibody IV-DI-35 (7), the ameba was identified as *N. fowleri*. Additionally, molecular analysis revealed that CDC: V 444 had sequence lengths for the ITS1, 5.8S, and ITS2 of 42 bp, 175, and 106 bp, respectively, and the sequence was identical to one of the two most common types in the United States (2). This clearly indicates that CDC: V 444 is not only *N. fowleri* but of a specific genotype that has been found commonly in the United States.

It is well known that *N. fowleri* causes an acute and fulminating PAM in humans (5), but it is not known to cause infections in other mammals, except for a paper that describes PAM in a South American tapir (4). Daft et al. reported that a total of 20 animals were suspected to have died from this infection in the summer of 1998. All of these animals belonged to a cattle ranch in San Bernardino, CA. Several animals died with similar symptoms in the summer of 1997 also (1). Further, according to one of the authors (B. Daft), specimens from three cases that were submitted to the California Animal Health and Food Safety Department in the summer of 1999 were also positive for *N. fowleri*. This report illustrates that PAM may not be as uncommon in animals as previously believed.

In this report, we have shown unequivocally that the ameba isolated from the cow brain (CDC: V 444) is *N. fowleri* based on sequence similarity with commonly found *N. fowleri* isolates from the United States. Two common *N. fowleri* types, characterized by ITS1 lengths of 42 bp and 86 bp, have been identified in the United States. Further, two other types, characterized by ITS1 lengths of 84 bp and 142 bp, have been identified in other parts of the world (2). The only type present in Australia, New Zealand, and Japan is characterized by a T-to-C transition in the 5.8S rRNA gene compared with the other types. This T-to-C transition 5.8S rRNA variant has been observed recently in the 42-bp type in the United States (8) as well but only in two strains that came from two different patients who had been swimming in the same source water but at different times. It is thus a very uncommon type. The *N. fowleri* strain (CDC: V 444) from the cow investigated here belongs to the common 42-bp type without the T-to-C transition in the 5.8S rRNA genes. The amplification here employed is robust enough to detect a specific genotype of *N. fowleri*. In a clinical setting, this genotypic tool can be useful in the diagnosis of a particular strain of *N. fowleri*. Further, it is invaluable in the surveillance and screening of environmental samples and helpful in the study of the distribution of particular isolates in nature.

Nucleotide sequence accession number. The sequence reported here was identical to GenBank/EMBL/DDBJ no. X96564 (2).

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